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Anti-tumor Activity of Arginine Deiminase from *Mycoplasma arginini* and Its Growth-inhibitory Mechanism

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Two kinds of arginine deiminase (AD, EC 3.5.3.6) were purified from cell extracts of *Mycoplasma arginini* (a-AD) and *Mycoplasma hominis* (h-AD), and their enzymic properties and anti-tumor activities were compared. The a-AD enzyme strongly inhibited the growth of mouse hepatoma cell line MH134 *in vitro*, and its concentration required for 50% growth inhibition (IC_{50}) was estimated to be about 10 ng/ml. The IC_{50} value of h-AD against the same cell line was estimated to be about 100 ng/ml, due to its low enzyme activity under the physiological pH condition, i.e., pH 7.4. These results show that the reaction pH profile of the a-AD was superior to that of the h-AD as an anti-tumor enzyme. Moreover, the effects of L-arginine metabolism-related substances on the anti-tumor activity of the a-AD were examined to study the growth-inhibitory mechanism of this enzyme. The addition of 2 or 4 m*M* L-arginine restored, in a dose-dependent manner, the growth of mouse MH134 hepatoma and Meth A fibrosarcoma cell lines that had been inhibited by 20 ng/ml of the a-AD. The addition of 2 or 4 m*M* L-ornithine, which is biosynthesized from L-arginine in the urea cycle and is the starting material in the polyamine-biosynthesis pathway, also partially restored it in a dose-dependent manner. These results indicate that the tumor cell growth inhibition caused by a-AD originates from the depletion of the essential nutrient L-arginine, and that the resulting block of the polyamine-biosynthesis pathway is involved in part in the inhibitory mechanism.

Key words: Arginine deiminase — Inhibitory mechanism — Tumor cell growth — *Mycoplasma*

Amino acid-degrading enzymes such as L-asparaginase (EC 3.5.1.1),[1,2] arginase (EC 3.5.3.1),[3,4] phenylalanine ammonialyase (EC 4.3.1.5),[5] and tryptophanase (EC 4.1.99.1)[6] inhibit the growth of tumor cells *in vitro* by depleting the respective amino acids in the culture medium. Among these enzymes, only L-asparaginase from *Escherichia coli* has been successfully used for clinical tumor therapy, but its use has been limited to some kinds of leukemia and lymphosarcoma.[7]

Arginine deiminase (AD, EC 3.5.3.6), which catalyzes the hydrolysis of L-arginine into L-citrulline and ammonia (Fig. 1), exists in various micro-organisms, such as *Streptococcus faecalis*,[8] *Pseudomonas putida*,[9] and non-glycolytic arginine-utilizing mycoplasmas.[10] Recently, we reported that AD from *Mycoplasma arginini* (a-AD) strongly inhibited the growth of various kinds of murine and human tumor cell lines *in vitro*,[11] and prolonged the survival time of mice implanted with four kinds of mouse tumor cell lines, i.e., hepatoma MH134, colon carcinoma Colon 26, sarcoma S-180, and melanoma B16.[12] Moreover, we found that the anti-tumor potency of a-AD was enhanced by chemical modification with polyethylene glycol, by improving its enzymic stability in the blood.[13] In another experiment, we established a high-level a-AD expression system in *E. coli* using recombinant DNA technology.[14] On the basis of these findings, we are interested in developing a-AD as a new anti-tumor drug.

However, the enzymic properties of a-AD as an anti-tumor agent have not been well studied, and the mechanism by which it causes inhibition of tumor cell growth is still unclear. Furthermore, the AD from *Mycoplasma hominis* (h-AD) has hardly been studied as regards its enzymic properties and anti-tumor potency, even though *M. hominis* is well known to display a high level of AD activity in its cells.[10,15] In this study, we prepared both a-AD and h-AD from the cell extracts of the respective *Mycoplasmas*. Their enzymic properties and anti-tumor potency were compared, and the superiority of a-AD as an anti-tumor enzyme was demonstrated. Moreover, we examined the effects of L-arginine metabolism-related substances (Fig. 1) on the anti-tumor activity of a-AD. The mechanism of the inhibition of tumor cell growth by this enzyme, is discussed.

MATERIALS AND METHODS

***Mycoplasmas* and mouse tumor cell lines** *M. arginini* (ATCC 23838) and *M. hominis* (ATCC 23114) were provided by the Institute for Fermentation, Osaka (IFO Nos. 14476 and 14850, respectively). Mouse hepatoma cell line MH134 was a generous gift from Dr. H. Taguchi, SRL Company, Tokyo; and mouse fibrosar-

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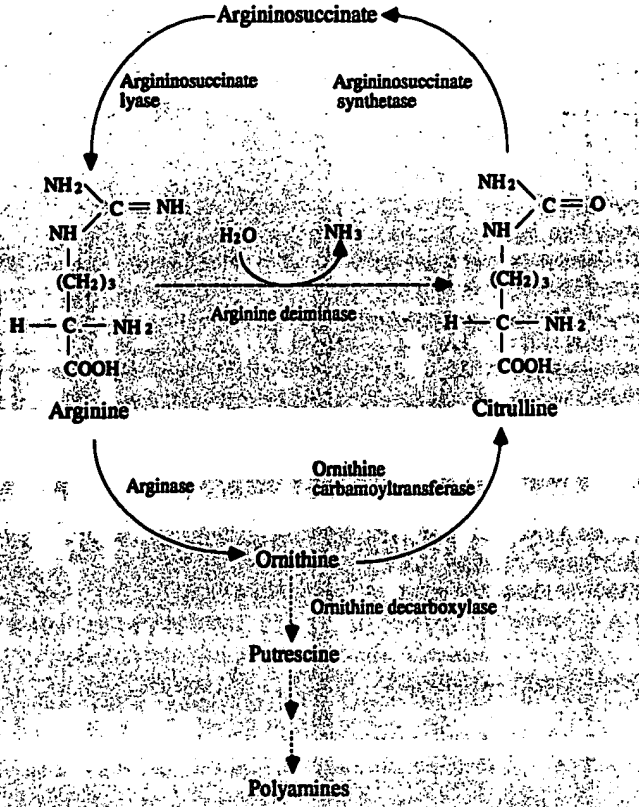

Fig. 1. Scheme of urea cycle (——), polyamine biosynthesis (- - -) and arginine deiminase reaction (— — —).

coma Meth A was kindly donated by Dr. T. Tashiro, Japanese Foundation for Cancer Research, Tokyo.

Assay of AD activity The AD activity was determined as described by Miyazaki *et al.*[11] The reaction mixture contained 10 m*M* L-arginine, 0.1 *M* buffer salts, and 0.1 ml of enzyme solution in a final volume of 1.0 ml. The buffer salts used were sodium acetate for pH 4.0–5.5, potassium phosphate for pH 6.0–7.5, and Tris-HCl for pH 8.0–9.0. This reaction mixture was incubated at 37°C for 5 min, after which time the reaction was terminated by the addition of 1.0 ml of a 1:3 mixture (v/v) of concentrated H_2SO_4 and concentrated H_3PO_4. The amount of L-citrulline formed during the incubation was determined by the measurement of a colored reaction product formed from L-citrulline and diacetyl monooxime according to the method of Oginski.[16] One unit of the AD activity was defined as the amount of the enzyme that converted 1 μmol of L-arginine to L-citrulline per minute under the assay conditions used. The protein concentration was determined by use of the Pierce BCA protein assay reagent with bovine serum albumin as a standard.

Purification of ADs from *Mycoplasmas* The a-AD enzyme was purified from the cell extract of a 2-liter culture of *M. arginini* as previously described.[12] The h-AD enzyme was also purified to homogeneity, as follows: *M. hominis* cells were cultured in 10 liters of Bacto PPLO broth w/o CV (Difco, Detroit, MI), pH 7.0, containing 20% horse serum (Irvine Scientific, Santana, CA), 2.5% yeast extract (Difco), and 1.0% L-arginine monohydrochloride. The cultured cells were collected by centrifugation at 15,000*g* for 20 min, washed twice with 100 ml of PBS (Ca^{2+}- and Mg^{2+}-free, phosphate-buffered saline), suspended in 10 ml of PBS containing protease inhibitors (50 μg/ml antipain-dihydrochloride, 40 μg/ml APMSF, 100 μg/ml chymostatin, 300 μg/ml E-64, 0.7 μg/ml pepstatin, and 500 μg/ml EDTA-Na_2; Boehringer Mannheim, protease inhibitors set), and sonicated at 20 kHz for 3 min in an ice bath. The sonicated cell suspension was centrifuged at 100,000*g* for 60 min at 4°C, and the resulting supernatant was subjected to purification by gel-filtration chromatography on a Sephacryl S-300 HR (Pharmacia, Uppsala, Sweden) column (4.4×100 cm), anion-exchange chromatography on a Q-Sepharose FF (Pharmacia) column (1.6×5 cm), and affinity chromatography on an Arginine-Sepharose 4B (Pharmacia) column (1.6×5 cm). The purity of the ADs was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% polyacrylamide gels by the method of Laemmli,[17] and the N-terminal amino acid sequences of the purified ADs were analyzed with a gas-phase protein sequencer (Applied Biosystems model 473 A).

Assay of growth-inhibitory activity The *in vitro* growth-inhibitory activity of the purified ADs was assessed in MH134 and Meth A cell lines. The tested cells (1.0×10^4 cells/well) were inoculated into the wells of 24-well microplates containing 1.0 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum (Irvine Scientific). Then 20 μl of sterilized enzyme solution was added to duplicate or triplicate wells to a final concentration of 5 to 25 ng/ml for a-AD and 50 to 200 ng/ml for h-AD, and the microplates were incubated at 37°C in a humidified 5% CO_2-95% air atmosphere. After 3 days in culture, the cells were counted with an automatic cell counter (Coulter, Hialeah, FL). In control cultures, the same volume of PBS was added to the well instead of the enzyme solution. When the effects of the addition of ammonia, L-citrulline, L-arginine, and L-ornithine were tested, 20 μl of their sterilized solutions was added to the well before cultivation.

RESULTS

Purification of h-AD The *M. hominis* cells (2.9 g wet weight) were obtained from 10 liters of their culture

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medium. The cell extract of *M. hominis* was fractionated by gel-filtration chromatography, and the AD activity was eluted in fractions corresponding to an apparent molecular weight of about 100,000 (data not shown). These fractions were then applied to the anion-exchange column. The h-AD was adsorbed on the column, and eluted with a linear gradient of 0–0.3 *M* NaCl. The AD activity was eluted at about 0.1 *M* NaCl, forming a single activity peak (data not shown). The peak fractions were pooled, and finally applied to the arginine-affinity column. The enzyme was adsorbed on the column, and eluted with a linear gradient of 0.2–1.0 *M* NaCl. The AD activity was eluted at about 0.6 *M* NaCl, forming a single protein peak (data not shown). By these 3 chromatographic steps, 4.3 mg of purified h-AD was obtained from the 10-liter culture of *M. hominis*, giving an activity yield of 51.8% and an 11.8-fold enrichment. However, when the cell extract of *M. hominis* was prepared without the protease inhibitors shown in "Materials and Methods," the AD activity was completely destroyed (data not shown).

Properties of a-AD and h-AD The purified a-AD and h-AD gave single bands with molecular weights of 45,000 and 47,000, respectively, on SDS-PAGE under non-reducing conditions (Fig. 2). Their electrophoretic mobilities were not affected by treatment with 2-mercaptoethanol, indicating that they contained no intermolecular disulfide bond (data not shown). The molecular weights of these enzymes in their native form were estimated to be about 90,000 by gel-filtration HPLC analyses (data not shown). These results suggest that both enzymes are dimeric proteins consisting of two identical subunits.

These enzymes (50 μg each) were subjected to sequencing on an automatic gas-phase protein sequencer, and the sequence of the first 30 amino acids from the N-terminal was determined for each protein (Fig. 3). The N-terminal amino acid sequences from 1 to 30 were

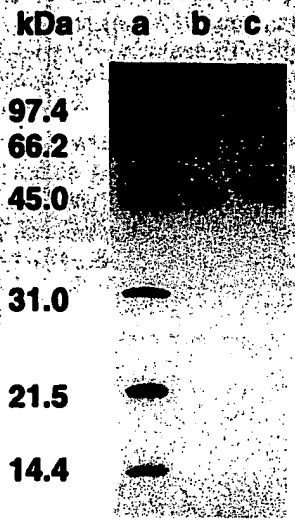

Fig. 2. SDS-PAGE analysis of a-AD (lane b) and h-AD (lane c) under non-reducing conditions. Purified a-AD (0.5 μg) or h-AD (0.5 μg) was put on each lane, and protein bands were stained with Coomassie Brilliant Blue R-250. Molecular weight markers (lane a) used were rabbit muscle phosphorylase B (Mr 97,400), bovine serum albumin (Mr 66,200), hen egg white ovalbumin (Mr 45,000), bovine carbonic anhydrase (Mr 31,000), soybean trypsin inhibitor (Mr 21,500), and hen egg white lysozyme (Mr 14,400).

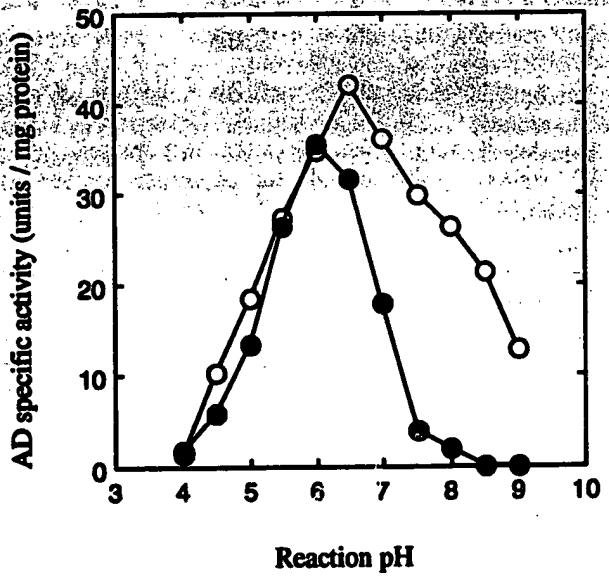

Fig. 4. Effects of reaction pH on enzyme activities of a-AD (○) and h-AD (●). Each point represents the average of duplicate assays.

```
          1                  10                  20                  30
M. arginini : S V F D S K F K G I H V Y S E I G E L E S V L V H E P G R E
M. hominis  : S V F D S K F N G I H V Y S E I G E L E T V L V H E P G R E
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Fig. 3. N-Terminal amino acid sequences of a-AD and h-AD.

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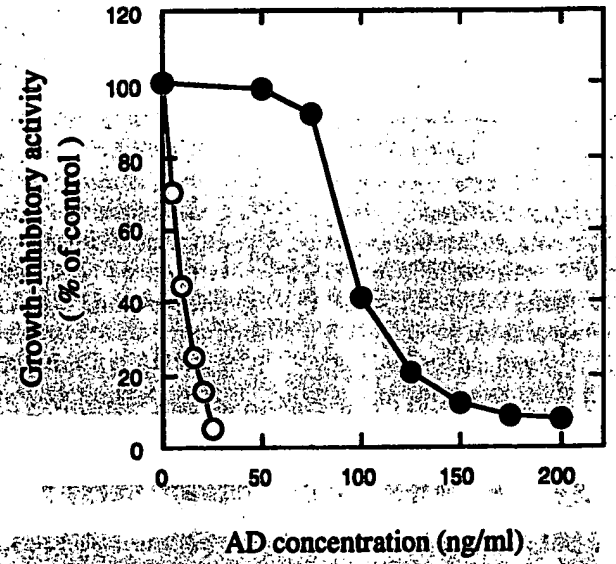

Fig. 5. Effects of various concentrations of a-AD (○) and h-AD (●) on the growth of MH134 cells. Each point represents the average of duplicate wells. The average cell number per well in the control cultures after 3 days was 1.70×10^6.

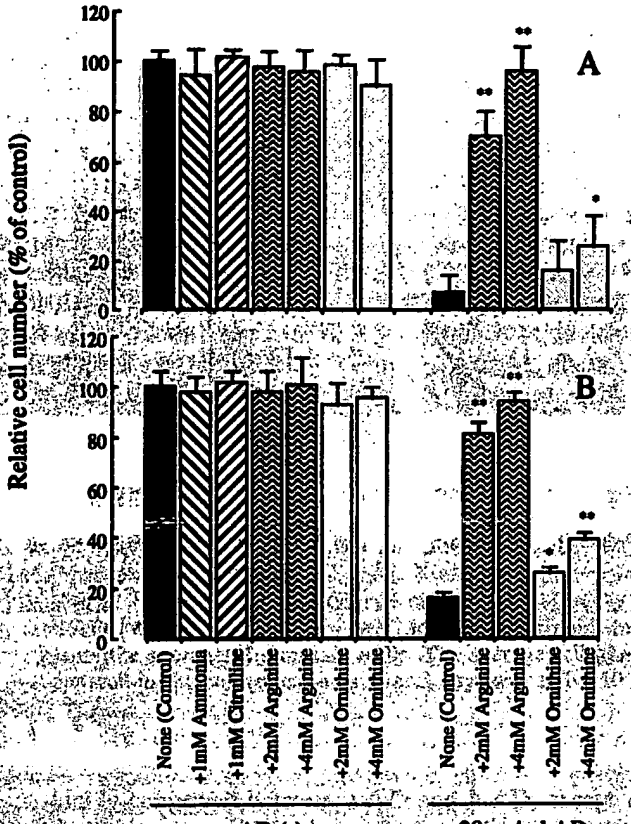

Fig. 6. Effects of ammonia, L-citrulline, L-arginine, and L-ornithine additions to the culture medium on the growth of MH134 (A) and Meth A (B) cells in the presence (20 ng/ml) or absence of a-AD. Each value represents the mean±SD for triplicate wells. The average cell numbers per well in the controls without a-AD after the 3-day culture were 1.69×10^6 (MH134) and 1.03×10^6 (Meth A). In the experiment with a-AD present, the significance of differences between data from control and additive-containing cultures was verified by the use of Student's *t* test: *; $P<0.05$, **; $P<0.01$.

highly conserved between a-AD and h-AD, except that the 8th amino acid (lysine and asparagine, respectively) and the 21st amino acid (serine and threonine, respectively) were different.

The a-AD exerted maximal enzyme activity (42.0 units/mg protein) at pH 6.5; and the h-AD, (35.5 units/mg protein) at pH 6.0 (Fig. 4). These enzymes showed almost the same reaction pH profiles from pH 6.0 to pH 4.0, that is, as the reaction pH became lower, the enzyme activities became weaker, in a linear fashion. But under the physiological pH condition, the former enzyme retained high activity (29.9 units/mg protein) at pH 7.4, while the latter enzyme retained little (3.9 units/mg protein) at pH 7.4.

Growth-inhibitory activities of a-AD and h-AD The *in vitro* growth-inhibitory activities of a-AD and h-AD against MH134 cell line are shown in Fig. 5. The a-AD strongly inhibited the growth of the tumor cells during the 3-day culture in a dose-dependent manner. The concentration required for 50% growth inhibition (IC_{50}) was estimated to be about 10 ng/ml. On the other hand, h-AD exerted poor growth-inhibitory activity against the same tumor cells, showing a sigmoidal dose-response curve, and its IC_{50} value was estimated to be about 100 ng/ml. In another experiment, these enzymes inhibited the growth of the Meth A cell line in a similar fashion; i.e., they gave almost the same dose-response curve as in the case of the MH134 cell line (data not shown).

Tumor cell growth-inhibitory mechanism of a-AD The effects of ammonia, L-citrulline, L-arginine, and L-ornithine addition to the culture medium on the growth of the MH134 and Meth A cell lines were tested in the presence or absence of a-AD (Fig. 6). In the absence of the a-AD, the addition of 1 m*M* ammonia or 1 m*M* L-citrulline, which are produced from L-arginine by the ADs (Fig. 1), had no effect on the growth of these tumor cell lines. Similarly, the addition of 2 or 4 m*M* L-arginine, which is the substrate of the enzyme, or L-ornithine, which is produced from L-arginine by arginase in the urea cycle and used for polyamine biosynthesis (Fig. 1), likewise showed no effect on cell growth. However, when 20 ng/ml a-AD was present in the culture medium, the

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growth of the tumor cell lines was strongly inhibited (the relative cell number was 7.5% of the AD(−) control for MH134 cells, and 17.0% of that control for Meth A cells). In the presence of 20 ng/ml a-AD, the addition of 2 or 4 m*M* L-arginine significantly restored, in a dose-dependent manner, the growth that had been inhibited by a-AD. When 4 m*M* L-arginine was added to the culture medium, the growth of both tumor cell lines completely recovered. In the case of 2 or 4 m*M* L-ornithine addition to MH134 culture, the growth inhibition of the tumor cell line caused by a-AD was partially reversed in a dose-dependent manner (but in the case of MH134 cells, significant restoration was observed only with 4 m*M* L-ornithine).

DISCUSSION

Recently, we reported the complete nucleotide sequence of the a-AD gene.[16] The sequence was different at four sites from that reported by Ohno *et al.*[19] We found that the a-AD gene comprised 1,230 bp encoding 410 amino acids, and the mature gene product contained 409 amino acids after elimination of the first methionine. The molecular weight of the a-AD was calculated to be 46,375 from the deduced amino acid sequence. Based on an earlier study, the complete nucleotide sequence of the AD gene of *M. hominis* PG21 (the same progenitor as ATCC 23114 used for this study) was reported by Harasawa *et al.*[19] The molecular weight of h-AD by our calculation from their data, 46,181, is similar to that of a-AD. The N-terminal amino acid sequence from 1 to 30 of each enzyme, analyzed by an automatic gas-phase protein sequencer (Fig. 3), corresponded exactly with the respective amino acid sequence deduced from the nucleotide sequence, and was highly conserved between a-AD and h-AD. Moreover, the isoelectric points of a-AD and h-AD were estimated to be pI 4.7 and pI 4.9, respectively, by isoelectric focusing analysis in our study (data not shown). These results show that the physicochemical properties such as molecular weight, N-terminal amino acid sequence, and isoelectric point are almost identical between the two AD's. However, it is notable that the reaction pH profile, which is an important enzymic property for an anti-tumor agent, as discussed later, was clearly different between these AD's (Fig. 4).

The enzyme activity of amino acid-degrading enzymes in the medium of tumor cell culture or in the blood of tumor patients depends on enzymic properties such as the Michaelis constant (K_m) and enzymic stability. For example, the K_m value of human liver arginase for L-arginine is 10.5 m*M*.[20] This K_m value seems too high to exert sufficient enzyme activity, as the L-arginine concentrations in the culture media and in blood are about 0.1 m*M*. In fact, it was previously reported that *in vitro* and *in vivo* tumor cell growth-inhibitory activities of that enzyme were very low or non-existent.[21] In contrast, the a-AD enzyme, which has a K_m value of about 0.2 m*M* with L-arginine, showed strong growth-inhibitory activity *in vitro*[11] and *in vivo*[12] in our previous studies. On the other hand, the stability of anti-tumor enzymes in the blood is also important. L-Asparaginase derived from *E. coli* has a half-life of 2.5–7.3 h in mouse blood, whereas that from yeast is completely inactivated within 1 h. Therefore the former enzyme exerts anti-tumor activity *in vivo*, but the latter one does not.[22] We reported that the half-life of a-AD in mouse blood, 4 h, was comparable to that of the *E. coli* L-asparaginase.[12] Moreover, its half-life was significantly prolonged by chemical modification with polyethylene glycol.[13] In the current study, it was confirmed that the reaction pH profile of anti-tumor enzymes is an important factor, as well as the K_m value and the enzymic stability in the blood. The a-AD maintained its enzyme activity at more than 70% of the maximal level at the physiological pH of 7.4, whereas the h-AD enzyme retained only about 10% of its maximal activity (Fig. 4). Therefore, a-AD strongly inhibited the growth of MH134 cells (its IC_{50} value was about 10 ng/ml), but h-AD showed poor growth-inhibitory activity (IC_{50} of about 100 ng/ml, Fig. 5). We also found that AD purified from *Pseudomonas putida* had maximal enzyme activity at pH 6.0, exerted negligible enzyme activity at pH 7.4 like h-AD, and showed very poor anti-tumor activity *in vitro* (data not shown). These results suggest that the enzymic properties of a-AD, such as the K_m value, enzymic stability in blood, and reaction pH profile, make this enzyme a suitable candidate for development as an anti-tumor agent.

To study the growth-inhibitory mechanism of a-AD, we examined the effects of the addition of ammonia and some amino acids to the culture medium (Fig. 6). The culture medium (RPMI 1640+10% fetal bovine serum) used for the tumor cells cultivation contains about 1 m*M* L-arginine, and little (trace) or no ammonia, L-citrulline, and L-ornithine. When the L-arginine is completely metabolized by the ADs, about a 1 m*M* concentration of each of ammonia and L-citrulline is produced in the culture medium. However, the addition of 1 m*M* ammonia or 1 m*M* L-citrulline had no growth-inhibitory effect on the tumor cells in the absence of a-AD. On the other hand, the addition of 2 or 4 m*M* L-arginine significantly restored the growth of tumor cells inhibited by 20 ng/ml AD in a dose-dependent manner, while its addition in the absence of AD had no effect on the tumor cell growth. These results indicate that the anti-tumor activity of a-AD is due to the depletion of L-arginine, not to the production of ammonia or L-citrulline. Interestingly, it was reported that polyamines play an important role

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in the proliferation and development of mammalian cells.[23,24] Therefore the polyamine-biosynthetic pathway (Fig. 1) represents an inviting target for the development of agents that inhibit carcinogenesis and tumor growth. Some inhibitors of polyamine production are currently available,[25] and other promising compounds are being tested as anti-tumor agents.[26] In our current study, the addition of 2 or 4 m*M* L-ornithine partially reversed the growth inhibition of tumor cells caused by a-AD in a dose-dependent manner, whereas its addition in the absence of the a-AD had no effect. These results indicate that L-ornithine, which is biosynthesized from L-arginine by arginase in the urea cycle, is also deficient in the culture medium due to the L-arginine depletion by the a-AD (Fig. 1). Moreover, it is suggested that the block of polyamine-biosynthesis caused by the deficiency of L-ornithine, the starting material for the polyamine-biosynthetic pathway, is related in part to the tumor cell growth-inhibitory mechanism of a-AD. However, further detailed studies are required to clarify the balance between the roles of depletion of the essential nutrient L-arginine and the block of polyamine-biosynthesis in the growth-inhibitory potency.

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